First evidence that intrinsic fetal heart rate variability exists and is affected by hypoxic pregnancy

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Key point summary

- We introduce a technique to test whether intrinsic fetal heart rate variability (iFHRV) exists and we show the utility of the technique by testing the hypothesis that iFHRV is affected by chronic fetal hypoxia, one of the most common adverse outcomes of human pregnancy complicated by fetal growth restriction.
- Using an established late gestation ovine model of fetal development under chronic hypoxic conditions, we identify iFHRV in isolated fetal hearts and show that it is markedly affected by hypoxic pregnancy.
- Therefore, the isolated fetal heart has intrinsic variability and carries a memory of adverse intrauterine conditions experienced during the last third of pregnancy.

Abstract

Fetal heart rate variability (FHRV) emerges from influences of the autonomic nervous system, fetal body and breathing movements, and from baroreflex and circadian processes. We tested whether intrinsic HRV, devoid of any external influences, exists in the fetal period and whether it is affected by chronic fetal hypoxia. Chronically catheterised ewes carrying male singleton fetuses were exposed to normoxia (n=6) or hypoxia (10% inspired O2, n=9) for the last third of gestation (105-138 dG; term~145 dG) in isobaric chambers. At 138dG, isolated hearts were studied using a Langendorff preparation. We calculated basal iFHRV matrix indices reflecting signal's variability, predictability, temporal symmetry, fractality and chaotic behaviour, from the systolic peaks within 15 min segments in each heart. Significance was assumed at $p<0.05$.. Hearts of fetuses isolated from hypoxic pregnancy showed approximately 4-fold increases in the Grid transformation as well as the AND similarity index (sgridAND) and a 4-fold reduction in the Scale dependent Lyapunov exponent slope. We also detected a 2-fold reduction in the Recurrence quantification analysis, percentage of laminarity (pL) and recurrences, maximum and average diagonal line (dlmax, dlmean) and the Multiscale time irreversibility asymmetry index. The iHRV measures dlmax, dlmean, pL and sgridAND correlated with left ventricular end-diastolic pressure across both groups (average R_2=0.38±0.03). This is the first evidence that iHRV originates in fetal life and that chronic fetal hypoxia significantly alters it. Isolated fetal hearts from hypoxic pregnancy exhibit a time scale dependent higher complexity in iFHRV.

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Non-standard Abbreviations and Acronyms

CIMVA, continuous individualized multi-organ variability analysis;

FHR, fetal heart rate;

FHR variability, FHRV;

iFHRV, intrinsic FHRV;

LVEDP, left ventricular end-diastolic pressure;

sgridAND, grid transformation AND similarity index

IUGR, intrauterine growth restriction

Introduction

Analysis of fetal heart rate (FHR) variability (FHRV) has served as a scientific and diagnostic tool to quantify the fluctuations of cardiac activity under various conditions since the early 1980's(Akselrod *et al.*, 1981). However, surprisingly, little is known about its biological origins. From studies of healthy adult subjects during exercise and investigations of heart-transplant recipients, the field is aware that intrinsic components of cardiac rhythm can contribute substantially to HRV(Akselrod *et al.*, 1981).

It is established that normal FHRV represents a complex, nonlinear integration of the activities of the sympathetic and parasympathetic nervous systems. Fetal body and breathing movements, sleep states(Nijhuis *et al.*, 1982) as well as baroreflex and circadian processes also influence FHRV(Dalton *et al.*, 1977; Visser *et al.*, 1982; Frasch *et al.*, 2009; Jensen *et al.*, 2009). There is some evidence for intrinsic pacemaker rhythms of the sino-atrial node that affect HRV in critically ill adult patients(Papaioannou *et al.*, 2013).

However, a prenatal origin of intrinsic influences in HRV has been difficult to prove. Dalton *et al.* reported that in chronically-instrumented fetal sheep *in vivo*, 35-40% of HRV remained after combined beta-adrenergic and parasympathetic blockade(Dalton *et al.*, 1983). Combined beta-adrenergic and parasympathetic blockade *in vivo* will not remove endocrine influences on the fetal heart mediated via non-autonomic agonists, nor mechanical influences, which will all still impose some variability *in vivo*. Further, if intrinsic HRV (iHRV) occurs, whether it is affected by chronic fetal hypoxia, one of the most common outcomes of human pregnancy complicated by fetal growth restriction, is completely unknown.

The isolated Langendorff *ex vivo* preparation of the fetal sheep heart is ideally suited for assessing iHRV because it is devoid of innervation or systemic hormonal influences. Therefore, the objectives of this work were to introduce to the field a new technique for physiological research and to show its utility by assessing whether iFHRV exists. Further, combining novel technology only recently available to induce chronic fetal hypoxia and fetal growth restriction in ovine pregnancy (Brain *et al.*, 2015*a*; Allison *et al.*, 2016), we tested

whether iFHRV is affected in the compromised IUGR fetus in late gestation. The data show that iFHRV exists and that it is affected by chronic fetal hypoxia. Therefore, these discoveries represent a significant conceptual advance in physiology. Further, the data expand technology to study the chronically hypoxic fetus and provide insight to improve fetal health surveillance.

Methods

Ethical Approval

All experiments were performed in accordance with the UK Home Office guidance under the Animals (Scientific Procedures) Act 1986 and were approved by the Ethical Review Board of the University of Cambridge.

Surgical Preparation

Briefly, chronically catheterized ewes carrying male singleton fetuses were exposed to normoxia (n=6) or hypoxia (10% inspired O2, n=9) for the last third of gestation (105-138 dG; term~145 dG) in bespoke isobaric chambers (Brain *et al.*, 2015*b*; Allison *et al.*, 2016; Shaw *et al.*, 2018) (Fig. 1 and 2). At 138dG, isolated hearts were studied under a Langendorff preparation using established techniques(Fletcher *et al.*, 2005; Niu *et al.*, 2013, 2018) (Fig. 3).

At 100±1 days gestational age (term ca. 145 days), pregnant Welsh mountain ewes carrying singleton pregnancies determined by ultrasound scan (Toshiba Medical Systems Europe, Zoetermeer, the Netherlands) underwent a laparotomy under general anaesthesia. In brief, food but not water was withdrawn for 24 h prior to surgery. Anaesthesia was induced by Alfaxan (1.5–2.5 mg kg^{-1} i.v. alfaxalone; Jurox Ltd., Worcestershire, UK) and general anaesthesia (1.5–2.0% isoflurane in 60:40 O_2:N_2O) maintained by use of a positive pressure ventilator (Datex-Ohmeda Ltd., Hatfield, Hertfordshire, UK). Antibiotics (30 mg kg^{-1} i.m. procaine benzylpenicillin; Depocillin; Intervet UK Ltd., Milton Keynes, UK) and an analgesic (1.4 mg kg^{-1} s.c. carprofen; Rimadyl; Pfizer Ltd., Kent, UK) were administered immediately before the start of surgery. Following a midline abdominal incision and uterotomy, the fetal hind limbs were exposed, and the fetal sex was determined. If male, then the fetuses were chosen for this study in order to control but not to address sex differences. Female fetuses were used for another experiment. The fetus was returned into the intrauterine cavity, and the uterine and maternal abdominal incisions were closed in layers. A Teflon catheter (i.d. 1.0 mm, o.d. 1.6 mm, Altec, UK) was then placed in the maternal femoral artery and extended

to the descending aorta, in addition to a venous catheter extended into the maternal inferior vena cava (i.d. 0.86 mm, o.d. 1.52 mm, Critchly Electrical Products, NSW, Australia). Catheters were filled with heparinised saline (80 I.U mL^{-1} heparin in 0.9% NaCl), tunnelled subcutaneously, and exteriorised via a keyhole incision made in the maternal flank to be kept inside a plastic pouch sewn onto the maternal skin. Inhalation anaesthesia was withdrawn, and the ewe was ventilated until respiratory movements were observed. The ewe was extubated when spontaneous breathing returned and moved into a recovery pen adjacent to other sheep with free access to food and water. A total of 15 Welsh Mountain ewes carrying male singleton fetuses were surgically instrumented for this study.

Postoperative care

Following surgery, ewes were housed in individual floor pens with a 12 h:12 h light:dark cycle and free access to hay and water. Antibiotics (30 mg kg^{-1} i.m. procaine benzylpenicillin; Depocillin; Intervet UK Ltd., Milton Keynes, UK) were administered daily to the ewe for 5 days following surgery. From 103 days of gestation, ewes were fed daily a bespoke maintenance diet made up of concentrate and hay pellets to facilitate the monitoring of food intake (Cambridge ewe diet: 40 g nuts kg^{-1} and 3 g hay kg^{-1}; Manor Farm Feeds Ltd.; Oakham, Leicestershire, UK). Generally, normal feeding patterns were restored within 24–48 h of recovery. On day 103 of gestation, ewes were randomly assigned to either of two experimental groups: normoxia (N: n = 6) or chronic hypoxia (H: n = 9) (Fig. 1).

Ewes allocated to chronic hypoxic pregnancy were housed in one of four bespoke isobaric hypoxic chambers (Telstar Ace, Dewsbury, West Yorkshire, UK; Fig. 2), as previously described (Brain *et al.*, 2015*a*; Allison *et al.*, 2016). In brief, chambers were supplied with variable amounts of nitrogen and air provided via nitrogen generators and air compressors, respectively, from a custom-designed nitrogen-generating system (Domnick Hunter Gas Generation, Gateshead, Tyne & Wear, UK). Ambient PO_2, PCO_2, humidity, and temperature within each chamber were monitored via sensors, displayed, and values recorded continuously via the Trends Building Management System of the University of Cambridge through a secure

Redcare intranet. In this way, the percentage of oxygen in the isolators could be controlled with precision continuously over long periods of time. For experimental procedures, each chamber had a double transfer port to internalise material and a manually operated sliding panel to encourage the ewe into a position where daily sampling of blood could be achieved through glove compartments (Fig. 2). Pregnancies assigned to the chronic hypoxia group were placed inside the chambers at 103 days of gestation under normoxic conditions (11 L sec^{-1} air, equating to 39.6 m^3 h^{-1}). At 105 days, pregnancies were exposed to approximately 10% O2 by altering the incoming inspirate mixture to 5 L sec^{-1} air: 6 L sec^{-1} N_2. A maternal arterial blood sample was taken daily to determine blood gas and acid base status, as described in detail before (Brain et al. 2015; Allison et al. 2016; Brain *et al.*, 2019). At 138 days of gestation, all animals were transferred to the *post mortem* laboratory. Ewes and their fetuses were humanely killed by overdose of sodium pentobarbitone (0.4 $ml.kg^{-1}$ I.V. Pentoject; Animal Ltd, York, UK) and the fetus exteriorized by Caesarean section. Pregnant ewes with male fetuses from the hypoxic chambers were transferred to the *post mortem* laboratory wearing a respiratory hood providing the same hypoxic mixture and underwent all procedures until isolation of the fetal heart under chronic hypoxic conditions.

Langendorff preparation

Fetal hearts were isolated, mounted onto a Langendorff apparatus and perfused at a constant pressure of 30 mmHg, as detailed by(Fletcher *et al.*, 2005) (Fig. 3). The ductus arteriosus was ligated. Pulmonary arteriotomy was performed. A recirculating solution of Krebs-Henseleit bicarbonate buffer containing ($mM.L^{-1}$) 120 NaCl, 4.7 KCl, 1.2 $MgSO_2.7H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 glucose, and 1.3 $CaCl_2.2H_2O$ was filtered through a 5 µm cellulose nitrate filter (Millipore, Bedford, MA, USA) and gassed with O_2:CO_2 (95:5) at 37^{o}C. A small flexible non-elastic balloon was inserted into the left ventricle through the left atrium. The balloon was filled with deionised water and attached to a rigid deionised water-filled catheter connected to a calibrated pressure transducer (Argon Medical Devices, Texas, USA). The balloon volume was set at 2.5 ml as a left ventricular end diastolic pressure (LVEDP) when the recording of approximately 5-10 mmHg in control hearts was obtained at this set value of the balloon volume. This allowed us to

calculate objectively the differences in pressure for the set volume(Niu *et al.*, 2013). After an initial 15 min stabilisation period, basal heart rate (HR), left ventricular systolic pressure (LVSP) and LVEDP were recorded. Basal left ventricular developed pressure (LVDP) was calculated as LVSP-LVEDP. The maximum and minimum first derivatives of the left ventricular pressure (dP/dt_{max} and dP/dt_{min}) were calculated using an M-PAQ data acquisition system (Maastricht Programmable AcQuisition System, Netherlands). For HRV analysis purpose, all original recording traces of left ventricular pressure were exported to LabChart® 7 software (ADInstruments, UK).

FHRV analysis

To derive FHRV, recordings of fetal left ventricular pressure sampled at 1kHz were analysed with the CIMVA (continuous individualized multiorgan variability analysis) software, as before (Durosier *et al.*, 2014). Inter-beat intervals were extracted from the pressure recordings using the systolic peaks. A range of 55 basal HRV indices was then calculated across five signal-analytical domains from the inter-beat interval time series, within 15 min segments in each heart, determined as an average of three non-overlapping 5 min intervals. We refer to Table 1 for details, where we provide the entire list of HRV indices calculated and their meaning in brief as it pertains to signal's variability, predictability, temporal asymmetry, fractality and chaotic behaviour.

Statistical analysis

Data are presented as Mean±SEM. The Student's *t* test for unpaired data was used to compare variables from hypoxic versus normoxic pregnancy. Relationships between variables were assessed by the Spearman rank correlation. Statistical significance was set at $P<0.05$ (SigmaStat).

Results

During the Langendorff preparation, the values for mean heart rate measured during basal conditions in normoxic and hypoxic fetuses did not differ between the groups (H: 153±7 and N:171±12 bpm). In contrast, the maximum and minimum derivatives of left ventricular pressure (dP/dt_{max} and dP/dt_{min}) were significantly reduced in hypoxic compared to normoxic fetuses ($p<0.05$, Fig. 4). Basal heart rate data were not available in these fetuses *in vivo*, prior to isolation of the fetal heart, as these preparations had maternal but not fetal surgical instrumentation.

Hearts isolated from chronically hypoxic fetuses showed distinct changes in iHRV measures reflecting chaotic and stochastic behaviours, recurrent plot behaviours such as periodicity, and fractality (degree of self-similarity) (Fig. 5; See Table 1 for terminology and supporting references). There were approximately 4-fold increases in the Grid transformation feature as well as the AND similarity index (sgridAND) and a 4-fold reduction in the Scale dependent Lyapunov exponent slope (SDLEalpha). We also detected a 2-fold reduction in the Recurrence quantification analysis, the percentage of laminarity and recurrences and maximum diagonal line (pL, pR, dlmax), the Multiscale time irreversibility asymmetry index (AsymI) and a 2-fold increase of Shannon Entropy (shannEn). There was also a moderate fall in the Detrended fluctuation analysis (area under the curve, DFA AUC). Of note, the conventional measures of HRV, such as RMSSD, HF or LF power were not different between the groups.

Combined, these data suggest that isolated fetal hearts from control pregnancy exhibited significant intrinsic FHRV. Further, based on the classification of HRV measures studied (Table 1), isolated fetal hearts from hypoxic pregnancy showed an overall higher complexity in iFHRV.

Measures of DFA AUC, dlmax, dlmean, pL, sgridAND and shannEn also correlated with LVEDP across both groups (Spearman R values of -0.579, -0.696, -0.546, -0.661, 0.639, and 0.554, respectively, or an

average R_2=0.38±0.03; Fig. 6). Measures of dP/dt_{max} and dP/dt_{min} correlated to SDLEalpha (Spearman R values of 0.571 and 0.607, respectively), sgridAND (Spearman R values of 0.800 and -0.539, respectively), dlmax (Spearman R values of 0.754 and 0.589, respectively). The iHRV measure dlmean also correlated to dP/dt_{max} (Spearman R value of 0.546); DFA AUC correlated to dP/dt_{min} (Spearman R value of 0.536). Overall, for dP/dt_{max} and dP/dt_{min} the average R_2=0.39±0.05.

Discussion

The maternal arterial blood gas data in this model of chronic hypoxia has been previously reported (Brain *et al.*, 2019). Measurements *in vivo* of maternal descending aortic blood samples show that exposure of pregnant sheep to a 10% inspired fraction of oxygen for a month in the last third of gestation, from 105 to 138 days of gestation (term is 145 days), led to a sustained controlled reduction in the maternal PaO_2 and in the percent saturation of haemoglobin (HbO_2) with oxygen. Chronic hypoxia also led to maternal respiratory alkalosis, with significant falls in maternal $PaCO_2$ throughout exposure. This model of maternal chronic hypoxia leads to significant intrauterine growth restriction (Brain *et al.*, 2019). In a separate study in which the fetus was also surgically prepared with catheters, we have previously reported that this level of maternal hypoxia for 10 days, from 125 to 135 days of gestation, also led to a significant reduction in the fetal partial pressure of oxygen in the descending aorta from 20.9±0.5 mmHg to 11.5±0.6 and in HbO_2 from 63.0±1.9 to 24.6±2.9% (both $p<0.05$; Allison et al. 2016). The data presented in this paper show that the fetal heart in late gestation has intrinsic influences, which may affect fetal cardiac function. Further, iFHRV is significantly affected by pregnancy complicated by chronic fetal hypoxia, of the type that leads to fetal growth restriction. Combined, these discoveries provide a conceptual advance to this field of study.

It is established that chronic hypoxia programmes an increased risk of diastolic dysfunction in the offspring and elevated LVEDP values are a sensitive measure of this cardiac dysfunctional phenotype (Wexler *et al.*, 1988; Xu *et al.*, 2006; Nagueh *et al.*, 2009; Giussani & Davidge, 2013). In the present study, we also found that myocardial contractility and relaxant capacity were significantly decreased in fetuses from chronic hypoxic pregnancy as shown by reduced dP/dt_{max} and dP/dt_{min}, respectively. Here, isolating the fetal heart of extrinsic influences in control and hypoxic pregnancy, we provide the first evidence to show that iHRV originates in fetal life and secondly that chronic fetal hypoxia significantly alters it. The significant relationship between nonlinear measures of FHRV and changes in dP/dt_{max} and dP/dt_{min} as well as LVEDP,

which is elevated in fetuses from hypoxic pregnancy (Niu *et al.*, 2018), suggests that such FHRV measures may reflect fetal myocardial dysfunction, both during cardiac systole as well as during diastole.

The findings raise several questions. What are the mechanisms contributing to iFHRV in the late gestation fetus in normal healthy pregnancy? What is the transfer mechanism by which *in utero* chronic hypoxia imprints upon iFHRV? May it be via impacting on myocardiogenesis, which then affects patterns of cardiac contractility and relaxant capacity, such as alterations in dP/dt_{max} and dP/dt_{min}? Does the putative transfer mechanism of *in utero* hypoxia upon iFHRV depend upon vagal and sympathetic fluctuations *in vivo* or is it entirely autochthonic, emerging from the adaptive processes within the excitatory cells themselves in response to chronic hypoxia?

Previous findings derived from sheep studies in which fetuses were subjected to a labour-like insult with worsening acidaemia and work in adult animal models of acidaemia indicate that around a pH of 7.2, the physiological myocardial activity is curbed via a Bezold Jarisch-like reflex. This is a vagally-mediated myocardial depressive reflex that reduces cardiac output under conditions of moderate acidaemia, thereby preserving depleted myocardial energy reserves (Harry *et al.*, 1971; Nuwayhid *et al.*, 1975; Nuyt *et al.*, 2001; Frasch *et al.*, 2008; Gold *et al.*, 2017). When labour is associated with worsening fetal acidaemia, fetal compensatory cardiovascular reflexes are sensitized(Thakor & Giussani, 2009) and at risk of becoming overwhelmed, leading to eventual cardiac decompensation and an increased risk of fetal brain injury (Yumoto *et al.*, 2005). Fetal acidaemia impacts upon fetal myocardial contractility, which further promotes decreased cardiac output and the inability to maintain fetal arterial blood pressure(Frasch *et al.*, 2008, 2011). It is not yet understood how fetal insults involving hypoxia with or without worsening acidaemia disrupt sinus node pacemaker activity, thereby affecting iFHRV. Fetal hypoxic environments trigger chronic sympathetic hyperactivity. The increasing beta-adrenergic drive on cardiac pacemaker cells synchronises their activity in acute mechanistic experiments, which would result in lower complexity and lower variability or lower temporal asymmetry (Yaniv *et al.*, 2014). It is interesting to consider what the identified

iFHRV features dlmax and sgridAND, which correlate to LVEDP, as well as dP/dt_{max} and dP/dt_{min}, may represent physiologically (cf. Table 1 for overview).

In general, interpreting the physiological meaning of each iFHRV measure is an inverse mathematical and physiological problem somewhat similar to interpreting an electroencephalogram, for example, because of its underlying spatiotemporal origins and physiological meaning behind each of its complex properties. In this context, we are bound by the intrinsic limitations of interpretability from the iFHRV, which is an indirect signal, until it is possible to also obtain the direct signal. The latter could be single and ensemble recordings of the electrical pacemaker cells, followed by their analysis and comparison to the indirect observations derived from HRV. We refer to (Kwan *et al.*, 2016; Herry *et al.*, 2019) where we introduce the notion of vagus code in vagus electroneurogram (*i.e.*, the direct observation) and attempt a more detailed discussion of the indirect observations. This is via HRV and comparing the proposed iHRV signature to HRV properties reflecting vagal denervation or a systemic response to a major physiological disruption by surgery. In the present manuscript, we address, at least in part, this limitation of indirect observation by relating the iFHRV measures influenced by chronic hypoxia to myocardial performance characteristics. This gives us a sense of potential usefulness of such iFHRV measures without breaking down each one of them physiologically. The latter may be intrinsically impossible due to the nonlinear nature of the underlying physiology, *i.e.*, several iFHRV measures may reflect one complex physiological process from different mathematical, signal-theoretical angles.

As to dlmax, it is derived from a recurrence plot where the diagonal lines represent the trajectory visiting the same region of the phase space at different times. The lengths of diagonal lines in a recurrence plot are related to the predictability of the system dynamics. Perfectly predictable systems would have infinitely long diagonal lines in the recurrence plot (high dlmax). Conversely, stochastic and chaotic systems would have very short diagonal lines (low dlmax)(Webber & Zbilut, 1994; Zbilut *et al.*, 2002; Webber & Marwan,

2015). In the present study, chronic hypoxic pregnancy reduced the dlmax component of iFHRV, which correlated to an elevated LVEDP compared to hearts isolated from normoxic fetuses.

The grid transformation AND similarity index (sgridAND) measures the dynamic system phase space reconstruction trajectory, with a specific embedding dimension and time delay. It is binarized over a grid (*i.e.*, pixel visited by the trajectory=1, all others=0) to produce an image. Two grid images corresponding to different time delays or different windows in time are then compared(Roopaei *et al.*, 2010*a*). The sgridAND measure is the normalized sum of the binary AND operation on the two compared images and represents a similarity index between the phase space trajectories from two consecutive windows. Low values indicate that the iFHRV dynamics have changed while high values mean the dynamics are similar or exhibiting a larger spread of trajectories, due, *e.g.*, to arrhythmia(Roopaei *et al.*, 2010*b*). This time scale dependent behaviour makes it difficult to simply state that complexity increased or decreased due to chronic hypoxia. Overall, a complexity increase was observed in most iFHRV measures. This is the case for the iFHRV calculated in hearts isolated from hypoxic fetuses (Fig. 7). Again, this correlates with greater resting LVEDP.

Combined, our findings indicate that *in utero* hypoxia reduces the short-term predictability of iFHRV and increases its long-range similarity. Both time scale dependent effects do not contradict each other because the effects are captured in different signal-analytical domains, one being a geometric feature of iFHRV and another referring to longer-term temporal processes in the informational domain (see also Table 1). Importantly, both changes occur with a consistent increase in LVEDP, demonstrating that complex iFHRV properties can be linked to a cardiac phenotype, in this case one of cardiac diastolic dysfunction.

Study limitations

This investigation was conducted in an ovine *ex vivo* fetal heart preparation. Albeit derived in one of the most appropriate animal species that shares similar temporal profiles of cardiovascular development to

humans(Morrison *et al.*, 2018), the present findings must be validated in human cohorts. This could be performed in the context of human heart transplants, which will likely require a multi-site effort, because it is rare, with ~10 transplants performed in the US per year (John & Bailey, 2018).

We did not measure the *in vivo* fetal heart rate values in the hypoxic and normoxic fetuses, as in these preparations only the mother but not the fetus was catheterised. Interestingly, in the *ex vivo* measurements, the values for fetal heart rate were not significantly different. Basal heart rate may influence the degree of HRV, but, at least based on our *ex vivo* observations, that was not a confounding factor in the present study (Monfredi *et al.*, 2014; Shaw *et al.*, 2018).

In this study, we calculated FHRV using a pressure signal from the *ex vivo* Langendorff preparation. This is in contrast with the *in vivo* FHRV studies where HRV is derived from the ECG. It is established that the temporal precision of the R peak detection is lower when the signal is triggered from the systolic blood pressure waveform peak compared to the signal being triggered from a 1000 Hz sampled ECG. Previously, we have reported in sheep and human fetuses (Durosier *et al.*, 2014; Li *et al.*, 2015) that the beat-to-beat variability derived from a 1000 Hz sampled ECG signal contains more predictive information than when it is being derived from a 4 Hz sampled signal. This is due to higher temporal precision of the R peak detection at the higher sampling rate. In this study, we used exclusively data recorded from pressure wave signal. Hence, significant changes that we detected at the lower sampling rate from pressure wave recordings are likely to be enhanced or at least reproducible at the higher resolution using ECG recording.

Understanding the relationship between iHRV and the cardiac function in vivo will require progressive validations.

Summary

We introduce a technique to the field of study that determines changes in iFHRV measures and validate the technique by showing that iFHRV exists in the late gestation fetus and that it can be significantly affected by chronic fetal hypoxia, providing physiological insight into the intrinsic control of cardiac function.

Competing interests: The authors have nothing to disclose.

Author contributions:
Y.N. and D.A.G. conceived and designed the experiments and carried out the analysis.
M.G.F. and C.L.H. carried out the analysis. All authors contributed to interpretation of the data and drafting of the manuscript. All authors contributed to critical revision and approved the final version of the manuscript.

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Tables

Table 1. Physiological classification of heart rate variability measures

Time domain measures:

Simple statistical description of variability in the time domain

Metric name	**Description and meaning**	**Meaning of change**	**Refs**
SDNN	Standard deviation of Normal-Normal (NN) intervals.	small = low variability	(Anon, 1996; Sassi *et al.*, 2015)
RMSSD	Root mean square of successive RR interval differences (beat-to-beat variance in HR). Estimates vagally mediated changes in HRV. Equivalent to Poincaré SD1	small = low variability	(Anon, 1996; Sassi *et al.*, 2015)
mDiff	Sample mean of the first difference of the RR interval time series.	small = low variability	(Anon, 1996; Sassi *et al.*, 2015)

CoV	Coefficient of variation: Standard deviation normalized by the mean	small = low variability	(Anon, 1996; Sassi *et al.*, 2015)
histSI	Similarity index between the statistical distribution of two consecutive data blocks	small = more complex	(Huang *et al.*, 2008)
Hjorth's Complexity	Measure of "excessive details with reference to sine wave", assesses complexity in the signal	small = less complex	(Hjorth, 1970, 1973)

Spectral content:

Quantification of the power in different frequency bands

Metric name	**Description and meaning**	**Meaning of change**	**Refs**
LF Power	Power contained in the low frequency band (0.04-0.2 Hz for fetal recordings) of the ECG spectrum. Reflects both sympathetic and vagal activity, and blood pressure regulation via baroreceptors.	small = less power	(Press & Rybicki, 1989;

			Anon, 1996; Sassi *et al.*, 2015)
HF Power	Power contained in the high frequency band (0.2-2 Hz for fetal recordings) of the ECG spectrum. Mostly reflects vagal modulation of HR and is related to the respiratory cycle.	small = less power	(Press & Rybicki, 1989; Anon, 1996; Sassi *et al.*, 2015)
LF/HF ratio	Represents sympathetic and parasympathetic modulation but its interpretation is experiment dependent and unclear in general.	small = less power	(Press & Rybicki, 1989; Anon, 1996; Sassi

			et al., 2015)
VLF Power	Power in the Very Low Frequency Band (0.003-0.04 Hz). Thought to relate to thermoregulation and to be sympathetically mediated	small = less power	(Press & Rybicki, 1989; Anon, 1996; Sassi *et al.*, 2015)

Poincaré plot analysis:

Recurrence map analysis with a delay of one Normal-Normal (NN) interval, *i.e.*, scatter plot of NN_{n+1} against NN_n

Metric name	**Description and meaning**	**Meaning of change**	**Refs**
SD1	Standard deviation perpendicular the line of identity. Represents short interval variations and reflects parasympathetic index of sinus node control. Equivalent to RMSSD.	small = low variability	(Brennan *et al.*, 2001;

			Sassi *et al.*, 2015)
SD2	Standard deviation along the line of identity. Represents long interval variation and is linked to both parasympathetic and sympathetic tones. Related to Standard Deviation	small = low variability	(Brennan *et al.*, 2001; Sassi *et al.*, 2015)
CSI	Cardiac Sympathetic Index: Ratio between SD2 and SD1, represents unpredictability of the RR time series	small = more random/scattered	(Toichi *et al.*, 1997)
CVI	Cardiac Vagal Index: Log of the Area of the ellipse fitted to Poincare plot i.e. total variability	small = low variability	(Toichi *et al.*, 1997)

Entropies:

Quantification of the amount and rate of production of information of a signal; assesses regularity/predictability

Metric name	Description and meaning	Meaning of change	Refs
shannEn	Shannon Entropy: Average information content of a signal	small = less complex	(Shannon, 1948)
QSE	Quadratic Sample Entropy: sample entropy normalized to standard deviation. Represents signal unpredictability/ regularity within short time segments	small = less complex	(Lake & Moorman, 2010)
Multiscale Entropy	Sample entropy over multiple scales	small = less complex	(Costa *et al.*, 2008)
KLPE	Kullback Leibler permutation entropy indicating deviation from randomness and predictability of the system.	small = more complex	(Frank *et al.*, 2006)
ARerr	Predictive error from an autoregressive model, assessed predictability	small = low variability	(Kampouraki *et al.*, 2009)

Indices of fractality:

Measures quantifying self-similarity and fractal- or multifractal-like behaviors.

Metric name	**Description and meaning**	**Meaning of change**	**Refs**
aFdP	Describes fractal-like point processes via a modified estimate of the Allan factor based on the average distance to a Homogeneous Poisson Point Process.	small = less complex	(Turcott & Teich, 1996)
fFdP	Describes fractal-like point processes via a modified estimate of the Fano factor, based on the average distance to a Homogeneous Poisson Point Process.	small = less complex	(Turcott & Teich, 1996)
IoV	Describes degree of variability at multiple time scales. For a self-similar process, IoV is equivalent to a Hurst parameter at multiple time scales.	small = less complex	(Lazarou *et al.*, 2009)
MultiFractal C1	Maximum of the multifractal spectrum that can be viewed as the most common variability within a window, similar to a global variability.	small = more variability	(Wendt *et al.*, 2007; Doret

			et al., 2011)
MultiFractal C2	Width of the multifractal spectrum i.e. how variability departs from C1 value. Small C2 = variability changes little over time.	small = less variability	(Wendt *et al.*, 2007; Doret *et al.*, 2011)
Correlation dimension	Overall complexity; related to fractal dimension; minimum number of variables required to characterize system dynamics.	small = less complex	(Grassberger & Procaccia, 1983)
DFA α_1	Scaling analysis method representing short-term correlation properties. Thought to reflect baroreceptor reflex and parasympathetic modulation.	`Rough ≈ 0.5` `Smooth ≈ 1.5` `healthy ≈ 1`	(Peng C-K *et al.*, 1993; Peng *et al.*, 1995;

			Delignieres *et al.*, 2006; Silva *et al.*, 2017)
DFA α_2	Scaling analysis method representing long-term correlation properties. Thought to reflect beat cycle regulatory mechanisms and autonomic modulation. Related to the Hurst exponent	`Rough ≈ 0.5` `Smooth ≈ 1.5` `healthy ≈ 1`	(Peng C-K *et al.*, 1993; Peng *et al.*, 1995; Delignieres *et al.*, 2006; Silva *et al.*, 2017)
DFA AUC	Area Under the DFA curve estimating the total variance of the signal across time scales	small = low variability	(Bravi *et al.*, 2011)

Power Law Slope	Slope of the linear portion of the power spectrum on a log-log plot. Represents long term scaling of fractal like processes with long range dependence (i.e. 1/f power-law exponent)	small = less complex	(Press & Rybicki, 1989; Anon, 1996; Delignieres *et al.*, 2006)
Hurst exponent	Index of long-range dependence and related to the fractal dimension of a system i.e. the minimum number of degrees of freedom of the dynamical system.	small = higher fractal dimension	(Delignieres *et al.*, 2006)
AsymI	Degree of temporal asymmetry and lack of invariance of the statistical properties of a signal; Pathologic signals are more symmetric than healthy ones.	small = less complex	(Costa *et al.*, 2008)

Chaos:

Quantification of chaotic and stochastic behaviors

Metric name	Description and meaning	Meaning of change	Refs
Largest Lyapunov exponent	Average exponential growth rate of the distance between 2 neighboring points in the dynamical system trajectory. Measure of predictability, entropy rate, chaotic behavior. Positive for chaotic data.	small = less complex/chaotic	(Rosenstein *et al.*, 1993; Sassi *et al.*, 2015)
SDLEalpha	Estimates the (negative) slope of Lyapunov exponents at multiple scales on a log-log plot. Characterizes the speed of loss of information i.e. the uncertainty involved in predicting the value of a random variable.	steeper slope = more complex	(Gao *et al.*, 2006, 2013; Hu *et al.*, 2010)
SDLEmax	Estimates the maximum of Lyapunov exponents across multiple scales. The maximum typically occurs at smaller scales and is related to entropy measures.	small = less complex	(Gao *et al.*, 2006, 2013; Hu *et al.*, 2010)

gcount	Trajectory of a dynamical system is represented on a discrete grid and the number of "visited" pixels is counted. Represents the degree of complexity or chaotic dimension.	small = less complex	(Roopaei *et al.*, 2010*b*)
sgridAND	Estimates similarity between discretized delayed versions of dynamical system trajectories. Measures spread of trajectories and their similarity between two time instants.	small = low variability	(Roopaei *et al.*, 2010*b*)

Nonlinear Energy operators:

Measures characterizing local energy distribution in a signal

Metric name	**Description and meaning**	**Meaning of change**	**Refs**
Teo	Teager energy operator average energy: Estimates local energy of a signal (related to that of a sine wave) and identifies portions with high energy.	small = low variability	(Kaiser, 1990; Ruffo *et al.*, 2010)

PSeo	Plotkin and Swamy energy operator average energy: Generalized version of the Teager's operator.	small = low variability	(Agarwal *et al.*, 1998)

Recurrence Quantification Analysis (RQA):

Quantification of patterns on a Recurrence Plot, which is a visualization of the recurrent states of a dynamical system. RQA assesses periodicity, chaos, laminarity and recurrences.

Metric name	**Description and meaning**	**Meaning of change**	**Refs**
pD	% of recurrent points forming diagonal lines, with a minimum of two adjacent points (deterministic). Measures predictability	small = more chaotic	(Webber & Zbilut, 1994; Marwan *et al.*, 2002)

pDpR	Ratio of %Determinism over %Recurrence. Quantifies transition/nonstationary periods in a system.	small = more stationary	(Webber & Zbilut, 1994; Marwan *et al.*, 2002)
pL	% of Laminarity i.e. laminar states (chaos-chaos transitions) and rapid changes in RR intervals	small = more complex	(Webber & Zbilut, 1994; Marwan *et al.*, 2002)
pR	% of recurrence (Global measure of recurrence). Periodic dynamics have higher % of recurrence than aperiodic dynamics.	small = more complex	(Webber & Zbilut, 1994; Marw

			an *et al.*, 2002)
dlmax	Max length of diagonal structures, representing exponential divergence of the trajectories. Detects transitions from periodic to chaotic behavior and Inversely related to largest Lyapunov exponent.	small= more chaotic	(Webber & Zbilut, 1994; Marwan *et al.*, 2002)
dlmean	Average length of the diagonal structures. Mean prediction time of the system.	small = more chaotic	(Webber & Zbilut, 1994; Marwan *et al.*, 2002)

tTime	Trapping time: Average length of vertical lines. Information about the frequency of the laminar states and their durations.	small = more chaotic	(Webber & Zbilut, 1994; Marwan *et al.*, 2002)
sedl	Shannon entropy of the diagonals. Rough measure of the information content of the trajectories (diagonal lines) on a Recurrence plot.	small = less complex	(Webber & Zbilut, 1994; Marwan *et al.*, 2002)
sevl	Shannon entropy of the vertical lines. Rough measure of the information content of the trajectories (vertical lines) on a Recurrence plot.	small = less complex	(Webber & Zbilut, 1994; Marw

			an *et al.*, 2002)
vlmax	Max length of vertical lines. Information about the time duration of the laminar states and marker of intermittency	small= more chaotic	(Webber & Zbilut, 1994; Marwan *et al.*, 2002)

Symbolic Dynamics

Description of a system's dynamics with a limited number of symbols, amounting to a coarse-graining of the dynamics and describing global short-time dynamics.

Metric name	**Description and meaning**	**Meaning of change**	**Refs**
SymDce_2	Modified conditional entropy: Characterizes entropy rate	small = less complex	(Voss *et al.*, 1996; Porta

			et al., 2001)
SymDfw_2	Number of forbidden words. A high number of forbidden words indicates a more regular behavior of time series	small = more complex	(Voss *et al.*, 1996; Porta *et al.*, 2001)
SymDp0_2	Patterns with no variation i.e. all the symbols are equal. Thought to reflect to cardiac autonomic modulation, predominantly sympathetic modulation.	small = more complex	(Voss *et al.*, 1996; Porta *et al.*, 2001, 2007; Guzzetti *et al.*, 2005)
SymDp1_2	Patterns with 1 variation i.e. two consecutive symbols are equal and the remaining one is different	small = less complex	(Voss *et al.*, 1996; Porta

			et al., 2001, 2007; Guzzetti *et al.*, 2005)
SymDp2_2	Patterns with 2 variations (either like or unlike variations). Thought to reflect to cardiac autonomic modulation, predominantly parasympathetic modulation.	small = less complex	(Voss *et al.*, 1996; Porta *et al.*, 2001, 2007; Guzzetti *et al.*, 2005)
SymDse_2	Shannon entropy of patterns: Characterizes entropy i.e. complexity of the pattern distribution.	small = less complex	(Voss *et al.*, 1996; Porta *et al.*, 2001,

2007;
Guzze
tti *et*
al.,
2005)

Figure legends

Figure 1. Experimental protocol for *ex vivo* analyses.

Figure 2. Isobaric hypoxic chambers and nitrogen-generating system. A specially designed nitrogen generating system (a) supplied variable amounts of compressed air and nitrogen to 4 bespoke isobaric hypoxic chambers housed in the hypoxic chamber laboratory (b and c). Each chamber was equipped with an electronic servo-controlled humidity cool steam injection system to return the appropriate humidity to the inspirate (i). Ambient PO_2, PCO_2, humidity and temperature within each chamber were monitored via sensors (ii). For experimental procedures, each chamber had a double transfer port (iii) to internalise material and a manually-operated sliding panel (iv) to bring the ewe into a position where daily sampling of blood could be achieved through glove compartments (v). Each chamber incorporated a drinking bowl on continuous water supply and a rotating food compartment (vi) for determining food intake. A sealed transfer isolation cart could be attached to a side exit (vii) to couple chambers together for cleaning.

Figure 3. Isolated Langendorff heart perfusion model.

Figure 4. The maximum and minimum derivatives of left ventricular pressure. Values are mean ± SEM. Groups are normoxic (N, n=6) and hypoxic (H, n=9). Significant differences are: * vs. N, $p<0.05$ (Student's t test for unpaired data).

Figure 5. Effects of chronic hypoxia during pregnancy on fetal intrinsic heart rate variability (iFHRV). All measures are listed alphabetically. See Table 1 for terminology. Values are mean ± SEM. Groups are normoxic (N, n=6) and hypoxic (H, n=9). Significant differences are: * vs. N, $P<0.05$; # $P<0.06$ (Student's t test for unpaired data).

Figure 6. Correlation of left ventricular end-diastolic pressure (LVEDP) and the intrinsic heart rate variability (iFHRV) measures dlmax and sgridAND. LVEDP in normoxic (empty circles) and hypoxic (black circles) groups. Spearman statistics: R_2=0.32, p=0.03, and R_2=0.63, p<0.001, respectively.

Figure 7. A representative beat-to-beat time series, followed by the corresponding grid transformation AND similarity index (sgridAND) and the recurrence plots are shown for a normoxic (right) and hypoxic (left) fetus to demonstrate iFHRV pattern differences revealed with such representation of the phase space organization of the beat-to-beat fluctuations.